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Induction of CIRBP expression by cold shock on bovine cumulus-oocyte complexes

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Contents

The aim of this study was to induce the cold-inducible RNA-binding protein (CIRBP) expression on cumulus-oocyte complexes (COCs) through exposure to a sub-lethal cold shock and determine the effects of hypothermic temperatures during the *in vitro* maturation of bovine oocytes. Nuclear maturation, cortical granule redistribution and identification of cold-inducible RNA binding-protein (CIRBP) were assessed after 24 h of *in vitro* maturation of control (38.5°C) and cold-stressed oocytes (33.5°C). Presence of CIRBP was assessed by Western Blot in COCs or denuded oocytes and their respective cumulus cells. Based on the odds ratio, cold-stressed oocytes presented higher abnormal cytoplasmic distribution of cortical granules and nuclear maturation than control group. Although, CIRBP was detected in both control and cold-stressed groups, cold-stressed COCs had 2.5 times more expression of CIRBP than control COCs. However, when denuded oocytes and cumulus cells were assessed separately, CIRBP only was detected in cumulus cells in both groups. In conclusion, cold shock induced CIRBP expression, but it negatively affected nuclear maturation and cortical granule distribution of bovine oocytes. Moreover, the expression of CIRBP was only identified in cumulus cells but not in oocytes.

1. Introduction

Cryopreservation of germplasm has become an essential part of the assisted reproductive techniques. These technologies allow conservation of animal genetic resources and preservation of the fertility in women. However, there are still some difficulties regarding the application of the cryopreservation methods on oocytes due to the large size and marked sensibility to cooling injuries of these cells (Sprícigo, Morais, Yang, & Dode, 2012).

Different strategies have been used to improve cryotolerance in mammalian oocytes through a temporary increase of general adaptation induced by sub-lethal stressors (Pribenszky et al. 2010) such as high hydrostatic pressure (Gu et al., 2017) and heat stress (Vendrell-Flotats, Arcarons, Barau, López-Béjar, & Mogas, 2017). In the same way, we hypothesized that exposure to low temperatures prior vitrification may induce cryotolerance in mammalian gametes and embryos.

The exposure to mild hypothermic temperatures induces the expression of cold-shock proteins (Liao, Tong, Tang, & Wu, 2017). CIRBP, also called CIRP and A18 hnRNP, is a constitutively expressed cold-shock protein highly conserved among different species whose expression is present in a large variety of tissues and cells, including the ovaries among others (Zhong & Huang, 2017). CIRBP is involved in several cellular processes such as cellular proliferation and cell survival and it is involved in anti-apoptotic and anti-senescence pathways (Liao et al., 2017; Zhong & Huang, 2017). These findings suggest that the induction of CIRBP during *in vitro* maturation (IVM) of oocytes could improve cryotolerance to vitrification procedures. For that reason, the aim of this study was to determine the responsiveness of bovine oocytes (*Bos taurus*) to differentially express CIRBP through hypothermic temperatures as a preliminary study before testing predicted CIRBP protective effects against oocyte vitrification.

2. Materials and methods

All experiments were performed according to the principles and guidelines of the Ethics Committee on Animal and Human Experimentation from the *Universitat Autònoma de Barcelona*.

2.1 Experimental design

Cumulus-oocyte complexes (COCs) were randomly distributed in two groups: control (C) and cold-stressed groups (CS). After 24 h of IVM, oocytes were fixed in paraformaldehyde (PFA) to evaluate nuclear maturation and cytoplasmic distribution of cortical granules (CGs). Additionally, COCs or denuded oocytes and their respective cumulus cells from both experimental groups were frozen at -20˚C for Western Blot analysis. Three independent biological replicates were performed in total.

2.2 *In vitro* maturation

COCs were collected by aspirating follicles from heifer ovaries after collecting them at a local slaughterhouse. After 3 washes in PBS supplemented with 0.5 mg/mL bovine serum albumin, 1 mg/mL glucose, 36 µg/mL pyruvate and 0.05 mg/mL gentamycin, groups of 50 oocytes were randomly placed in 500 µL maturation medium in four-well dishes and cultured for 24 h at 38.5˚C (C) or 33.5˚C (CS) in independent incubators under an atmosphere of 5% CO_2 in humidified air. The maturation medium was composed by TCM-199 supplemented with 10% foetal calf serum and 10 ng/ml epidermal growth factor.

2.3 Assessment of nuclear maturation and cortical granule distribution

COCs were denuded of cumulus cells by gentle pipetting. Nuclear maturation was assessed as the percentage of oocytes that have reached the metaphase II stage by checking the extrusion of the first polar body. The zona pellucida was dissolved using a solution containing 0.4% pronase for 8 min. Oocytes were then fixed in 4% PFA (45 min, room temperature), permeated (0.3% Triton-X100, 30 min, room temperature) and stained (100µg/mL fluorescein isothiocyanate-labeled *Lens culinaris* agglutinin) as previously described by Andreu-Vázquez et al. (2010). Oocytes were transferred to mounting medium containing DAPI (Vector labs, Burlingame, CA, USA) and coverslipped. CGs distribution was classified into four patterns according to the classification of Hosoe & Shioya (1997) modified by Andreu-Vázquez et al. (2010) (pattern I: distribution in clusters - immature CGs distribution; pattern II: individually dispersed and partially clustered - incomplete CGs distribution; pattern III: distributed beneath the plasma membrane - optimal CGs distribution; pattern IV: no CGs - over matured).

2.4 Western blotting for CIRBP

Western blotting (WB) was performed following the described protocol by Alvarez-Rodriguez, López-Béjar, & Rodriguez-Martinez (2019). Briefly, COCs or denuded oocytes and their respective cumulus cells were homogenized by sonication in commercial lysis buffer (RIPA) at 4˚C. Protein concentration was determined by the DC™ Protein Assay kit (Bio-Rad), with bovine serum albumin as standard. Then, 25 µg of each sample were mixed with 4x sample buffer and heated for 10 minutes at 70 °C.

Extractions were loaded into 4%-20% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. For protein identification, membranes were blocked at room temperature for 60 min and incubated overnight at 4°C with rabbit monoclonal anti-CIRBP antibody [EPR18783] (ab191885, Abcam) at dilution 1/500. To standardize the results, a polyclonal IgG anti-α-Tubulin antibody (Sigma) was used at a dilution 1/1,000 in the same membranes. To visualize immunoreactivity, membranes were incubated 60 min at room temperature with secondary antibody anti-rabbit horseradish peroxidase conjugated (31460, Pierce Biotechnology) at dilution 1/10,000. After scanning by FluorChem® HD2 (Alpha Innotech), optical density was quantified by ImageJ Software.

2.5 Statistical analysis

The R Software (version 3.4.4) was used for data analysis. Replicate (1-3), group (C and CS), extrusion of first polar body (matured and non-matured) and CGs distribution (pattern I and III) were recorded for each oocyte. Three logistic regression analyses were performed in total using the nuclear maturation state or the CGs distribution data as dependent variables (0 and 1) in each individual analysis. Replicate and group were used as independent factors in each analysis. Intensity of CIRBP bands in WB were analysed by t-test comparing C with CS groups.

3. Results

Based on the odds ratio, the likelihood for an oocyte of showing CGs distribution pattern I (immature CGs distribution) was 9.75 times higher for CS than for C ($p < 0.05$). For CGs distribution pattern III (optimal CGs distribution), the likelihood to show

non-optimal distribution pattern was 5.6 times greater for CS than for C ($p < 0.05$). The risk to undergo anomalous nuclear maturation was 2.72 times higher in CS oocytes than C ones ($p < 0.05$) (Figure 1).

CIRBP expression was detected in both C and CS groups. Significantly higher ($p < 0.05$) levels of intensity were observed in CIRBP bands of CS compared with C in COCs analysis (Figure 2 and Figure 3). When oocytes were denuded, no expression of CIRBP was detected in oocytes while their respective cumulus cells showed CIRBP expression in both C and CS groups (Figure 2).

4. Discussion and conclusions

To our knowledge, this is the first study to describe the differential expression of CIRBP on bovine COCs after IVM in sub-lethal cold-shock-induced conditions as well as its effect on oocyte nuclear maturation and cytoplasmic distribution of CGs. According to our results, cold shock appears to negatively affect the optimal competence of oocytes regarding nuclear maturation and cytoplasmic CGs distribution. In addition, cold shock induced an increase of CIRBP expression on COCs. The increase of CIRBP in cumulus cells could play important roles in cryoprotective protection of oocytes through the interaction between cumulus cells and oocytes (Komatsu & Masubuchi, 2018). However, little is known about the relationship between CIRBP expression and the developmental competence of bovine vitrified-warmed oocytes. In this way, the developmental competence of vitrified-warmed yak oocytes (*Bos grunniens*) was improved by an increase of CIRBP (Pan et al., 2015). Taking together, new approaches should be performed to clarify the role of CIRBP on bovine COCs. Moreover, further studies are needed to apply the differential expression of

CIRBP in cumulus cells into an effective tool for improving vitrification cryotolerance minimizing the intrinsic negative effects of cold shock during IVM of bovine oocytes.

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Conflict of Interest Statement

None of the authors have any conflict of interest to declare.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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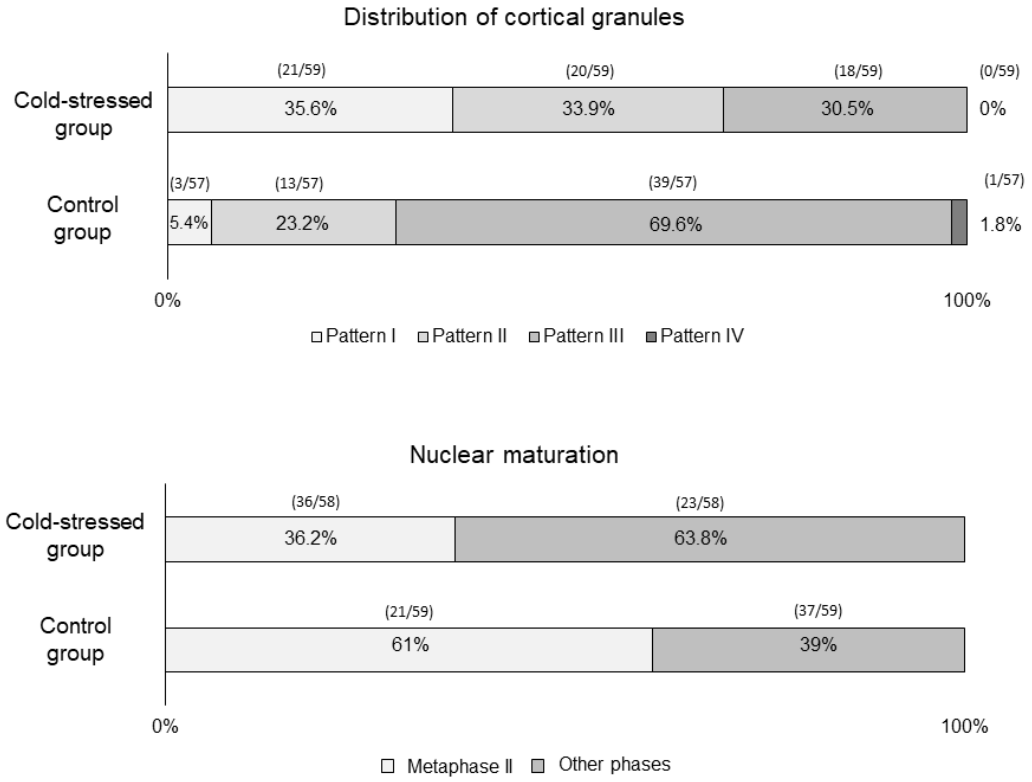
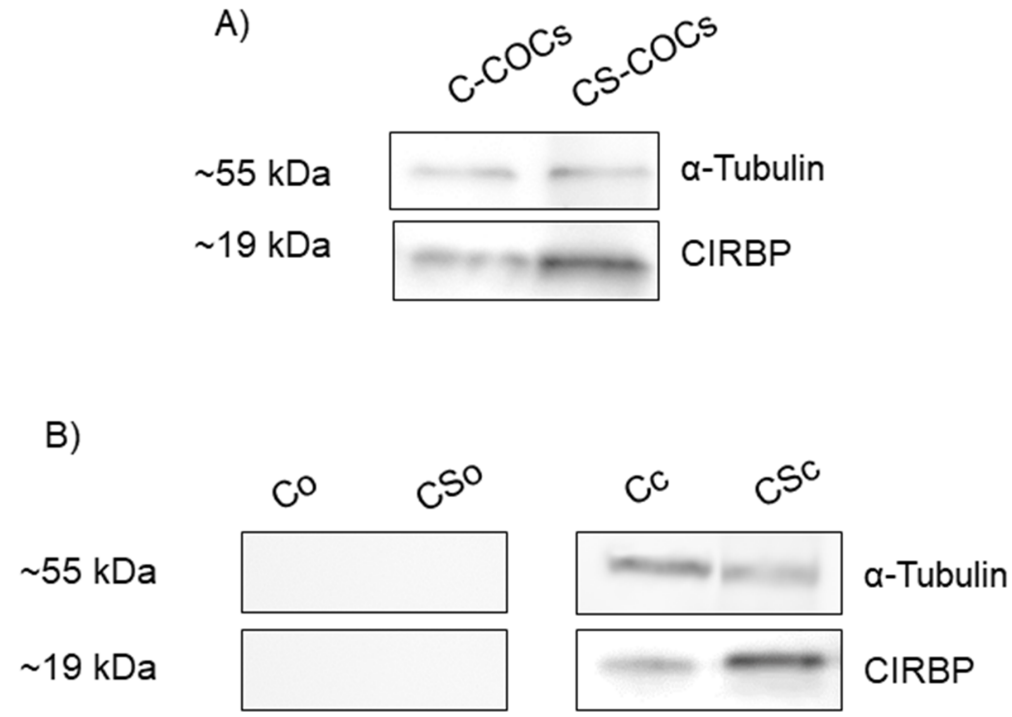


212

213 Figure 1. Distribution of cortical granules (CGs) and nuclear maturation of cold
214 stressed-oocytes (n=59 and n=58, respectively) and control oocytes (n=57 and n=59,
215 respectively) during 24 h of *in vitro* maturation. CGs distribution were distributed into
216 four patterns according to the classification of Hosoe & Shioya (1997) modified by
217 Andreu-Vázquez et al. (2010) (pattern I: distribution in clusters - immature CGs
218 distribution; pattern II: individually dispersed and partially clustered - incomplete CGs
219 distribution; pattern III: distributed beneath the plasma membrane - optimal CGs
220 distribution; pattern IV: no CGs - over matured). Nuclear maturation was classified as
221 the extrusion of the first polar body.



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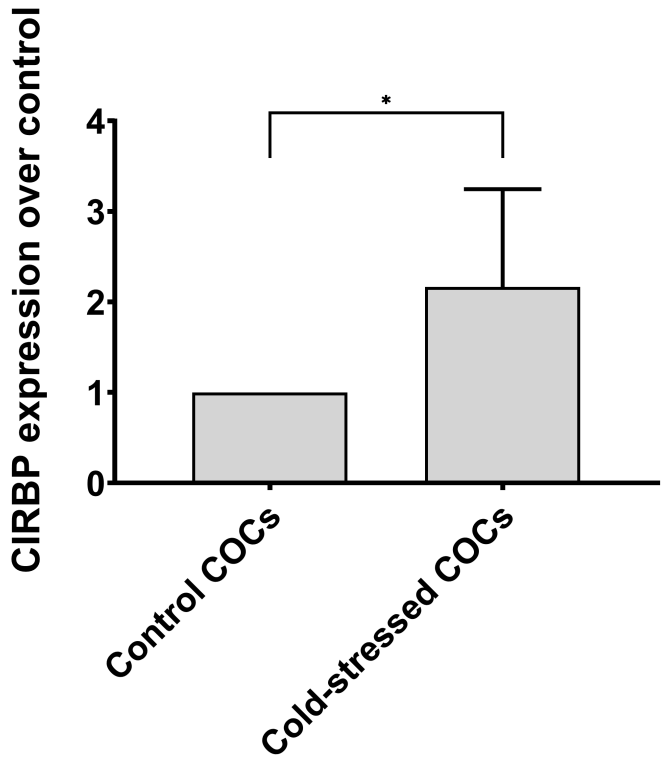
223 Figure 2. Analysis of the presence of CIRBP (19 kDa) by Western Blotting (WB) in
224 cumulus-oocyte complexes (COCs), and denuded oocytes and their respective cumulus.
225 Oocytes were *in vitro* matured at 38.5˚C (control group) or at 33.5˚C (cold-stressed
226 group). Membrane A: WB of COCs; membrane B: WB of denuded oocytes and their
227 respective cumulus cells. C-COCs: control COCs, CS-COCs: cold-stressed COCs, Co:
228 control oocytes, Cc: control cumulus cells, CSo: cold-stressed oocytes, CSc: cold-
229 stressed cumulus cells.



230

231 Figure 3. Relative expression (mean ± SD) of CIRBP protein in cumulus of bovine

232 cumulus-oocyte complexes (COCs) in control and cold-stressed groups. Three

233 independent blots were used for relative quantification. The control group matured at

234 38.5°C was used as calibrator. Different letters on the bars indicate values that differed

235 significantly ($p < 0.05$).